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Temporal Changes in PTEN and mTORC2 Regulation of Hematopoietic Stem Cell Self-Renewal and Leukemia Suppression

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SUMMARY

Pten deletion from adult mouse hematopoietic cells activates the PI3-kinase pathway, inducing hematopoietic stem cell (HSC) proliferation, HSC depletion, and leukemogenesis. *Pten* is also mutated in human leukemias, but rarely in early childhood leukemias. We hypothesized that this reflects developmental changes in PI3-kinase pathway regulation. Here we show that *Rictor* deletion prevents leukemogenesis and HSC depletion after *Pten* deletion in adult mice, implicating mTORC2 activation in these processes. However, *Rictor* deletion had little effect on the function of normal HSCs. Moreover, *Pten* deletion from neonatal HSCs did not activate the PI3-kinase pathway or promote HSC proliferation, HSC depletion, or leukemogenesis. *Pten* is therefore required in adult, but not neonatal, HSCs to negatively regulate mTORC2 signaling. This demonstrates that some critical tumor suppressor mechanisms in adult cells are not required by neonatal cells. Developmental changes in key signaling pathways therefore confer temporal changes upon stem cell self-renewal and tumor suppressor mechanisms.

INTRODUCTION

Hematopoietic stem cells (HSCs) persist throughout life by undergoing self-renewing divisions regulated by networks of proto-oncogenes and tumor suppressors (He et al., 2009). Mechanisms that promote HSC self-renewal are commonly overactivated or ectopically activated in leukemia cells (Reya et al., 2001). These self-renewal mechanisms are co-opted by leukemia cells irrespective of whether they arise from HSCs or from restricted progenitors (Krivtsov et al., 2006).

HSC properties and self-renewal mechanisms change over time. The transcription factor Sox17 is required for the maintenance of fetal and neonatal, but not adult, HSCs (Kim et al., 2007). Conversely, the epigenetic/transcriptional regulators Bmi-1, Tel/Etv6, and Gfi-1 regulate the maintenance of postnatal, but not fetal, HSCs (Lessard and Sauvageau, 2003; Park et al., 2003; Hock et al., 2004a, 2004b). The temporal changes in HSC self-renewal mechanisms raise the question of whether the driver mutations that are competent to cause leukemia also change over time.

Hematopoietic malignancies occur throughout life and the incidence of specific driver mutations varies with patient age, even within the same subtype of leukemia (Downing and Shannon, 2002). Myeloid leukemias in young children are often driven by translocations involving transcription factors (Downing and Shannon, 2002). In contrast, myeloid leukemias in older children and adults are much more likely to have mutations that activate the PI3-kinase pathway, including *Bcr-Abl* and *Flt3* internal tandem duplications (Meshinchi et al., 2001; Zwaan et al., 2003; Martelli et al., 2006; Kharas et al., 2008, 2010). These observations raise the question of whether age-dependent changes in leukemia driver mutations occur because of developmental changes in signaling mechanisms that regulate self-renewal.

The *Pten* tumor suppressor negatively regulates PI3-kinase pathway signaling and is commonly inactivated in many cancers (Di Cristofano and Pandolfi, 2000). *Pten* loss-of-function mutations occur frequently in T-precursor ALL (T-ALL) and *Pten* is inactivated in some acute myeloid leukemias (AMLs) by mutations, promoter methylation, or transcriptional repression (Dahia et al., 1999; Gutierrez et al., 2009; Larson Gedman et al., 2009; Yoshimi et al., 2011). While B-ALL usually presents in young children and rarely involves *Pten* deletion (Mullighan et al., 2011), pediatric T-ALL exhibits an older mean age of presentation (~9–10 years of age) (Smith et al., 1999; Clappier et al., 2007; Karrman et al., 2009) and commonly involves *Pten* deletion or other mutations that activate PI3-kinase pathway signaling (Gutierrez et al., 2009).

One possibility is that PI3-kinase pathway regulation changes over time in hematopoietic cells. *Pten* deletion from hematopoietic cells in adult mice leads to the development of T-ALL (Yilmaz

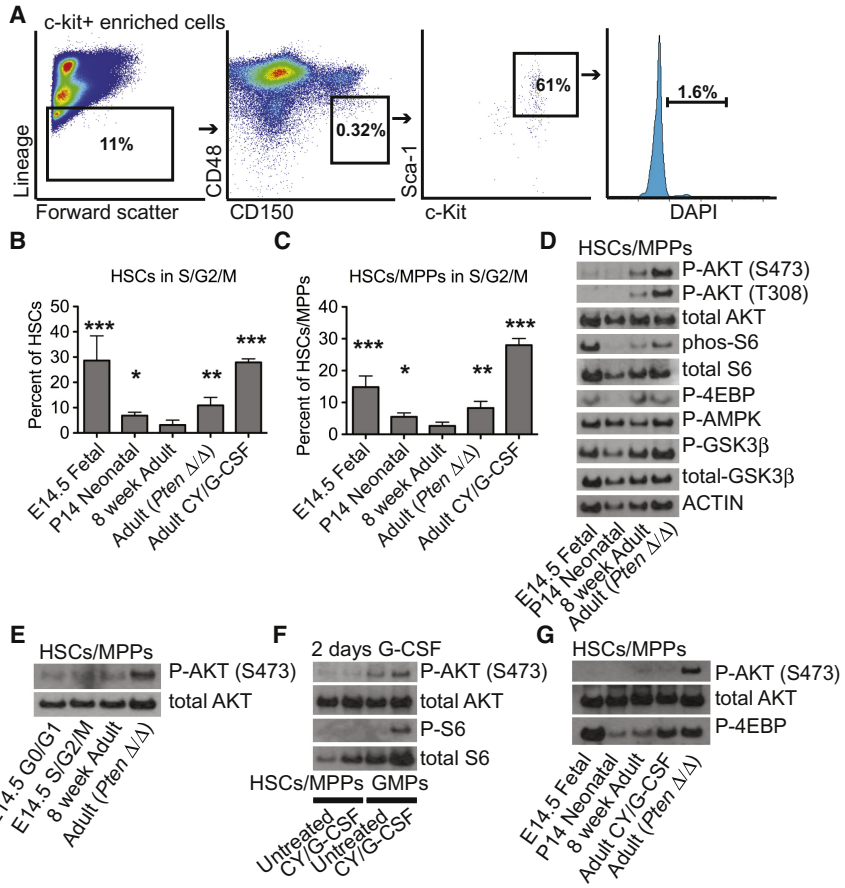

Figure 1. HSCs that Proliferate under Physiological Conditions Exhibit Little or No Increase in AKT Phosphorylation

(A) Representative flow cytometry plots for HSC cell cycle analysis.

(B) The percentage of $CD150^{+}CD48^{-}$LSK HSCs in S/G2/M phases of the cell cycle from E14.5 fetal (n = 3), P14 neonatal (n = 3), 8-week-old adult (n = 6), 8-week-old pIpC-treated *Mx1-Cre*; $Pten^{fl/fl}$ (n = 3), and cyclophosphamide/G-CSF-treated adult mice (n = 3). All comparisons are relative to 8-week-old adult mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars reflect standard deviation and p values were calculated by two-tail Student's t test.

(C) $CD48^{-}$LSK HSCs/MPPs have a similar cell cycle distribution to that of HSCs.

(D) PI3-kinase pathway components in HSCs/MPPs (30,000 $CD48^{-}$LSK cells per lane).

(E) AKT phosphorylation in fetal HSCs in G0/G1 versus S/G2/M phases of the cell cycle (10,000 cells per lane).

(F) Adult GMP myeloid restricted progenitors, but not HSCs/MPPs, exhibited increased AKT and S6 phosphorylation after cyclophosphamide/2 days G-CSF treatment (20,000 cells per lane).

(G) Even after cyclophosphamide and 4 days of G-CSF, mobilized spleen HSCs/MPPs (20,000 cells per lane) did not exhibit increased AKT phosphorylation relative to normal adult HSCs/MPPs.

et al., 2006; Zhang et al., 2006; Guo et al., 2008; Lee et al., 2010). *Pten* deletion from adult mouse HSCs causes these cells to go into cycle and to be depleted by a tumor suppressor response induced by hyperactivation of mTOR (Yilmaz et al., 2006; Lee et al., 2010). Both leukemogenesis and HSC depletion after *Pten* deletion can be rescued by treating mice with rapamycin (Yilmaz et al., 2006). This suggests that these phenotypes are mediated by RAPTOR/mTORC1 activation; however, rapamycin can also indirectly inhibit RICTOR/mTORC2 signaling (Sarbassov et al., 2006), and mTORC1 activation is influenced by mTORC2 signaling (Guertin and Sabatini, 2007; Guertin et al., 2009). Consequently, the relative contribution of mTORC1 and mTORC2 to these phenotypes is uncertain. It also remains unclear whether *Pten* deletion drives HSCs into cycle by activating physiological mechanisms that induce HSC division or by activating mechanisms that do not normally regulate HSC division.

To address these questions we evaluated PTEN function, RICTOR/mTORC2 function, and PI3-kinase pathway activation in fetal, neonatal, and adult HSCs. AKT phosphorylation did not detectably increase in HSCs that divided under physiological conditions. Consistent with this, *Rictor* deletion had little effect on HSC frequency or function. However, *Rictor* deletion largely rescued the increased AKT phosphorylation, HSC depletion, and leukemogenesis observed after *Pten* deletion from adult HSCs. This suggests that *Pten* deletion promotes HSC proliferation by activating signaling pathways that have a limited role in HSC self-renewal under physiological conditions. The observation that *Pten* deletion provides no selective advantage to neonatal hematopoietic cells may explain why *Pten* mutations are rare in leukemias that affect young children. Temporal changes in the signaling mechanisms that regulate self-renewal lead to temporal changes in leukemogenic mechanisms.

RESULTS

Normal HSC Proliferation Is Not Associated with Increased AKT Phosphorylation

HSCs divide rapidly during fetal and neonatal development (Morrison et al., 1995) and after cyclophosphamide/G-CSF treatment (Morrison et al., 1997). We compared the cell cycle status of HSCs ($CD150^{+}CD48^{-}Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$) isolated from embryonic day (E)14.5 fetal mice, postnatal day (P)14 neonatal mice, 8-week-old adult mice, and mice that were treated with cyclophosphamide and two daily doses of G-CSF (Figures 1A and 1B; Table S1 available online). We also administered poly-inosine:poly-cytosine (pIpC) to *Mx1-Cre; Pten*$^{fl/fl}$ mice 6 weeks after birth and isolated HSCs for cell cycle analysis at 8 weeks after birth. The fraction of HSCs in S/G2/M phases of the cell cycle decreased significantly from E14.5 to P14 to 8 weeks after birth (Figure 1B). The frequency of HSCs in S/G2/M phases of the cell cycle after cyclophosphamide/G-CSF treatment was significantly greater than after *Pten* deletion (Figure 1B).

To assess whether AKT activation correlates with the frequency of HSCs/MPPs in cycle, we compared AKT phosphorylation between $CD48^{-}Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ ($CD48^{-}$LSK)

HSCs/MPPs (Kiel et al., 2005; Kim et al., 2006) from E14.5 fetal liver, P14 bone marrow, 8-week-old adult wild-type bone marrow, and 8-week-old bone marrow after *Pten* deletion from *Mx1-Cre; Pten*$^{fl/fl}$ mice. The cell cycle distribution in the HSC/MPP population was similar to that of highly purified HSCs (Figure 1C), consistent with prior studies (Foudi et al., 2009). As expected, AKT and S6 were more highly phosphorylated in *Pten*-deficient adult HSCs/MPPs as compared to wild-type adult HSCs/MPPs (Figure 1D). However, phosphorylated AKT levels were lower in fetal and neonatal HSCs/MPPs compared to normal adult HSCs/MPPs (Figure 1D), even though fetal and neonatal HSCs/MPPs were more mitotically active (Figure 1C). Levels of phosphorylated S6 were higher in fetal HSCs/MPPs but lower in neonatal HSCs/MPPs as compared to adult HSCs/MPPs (Figure 1D). Phosphorylated AMPK and GSK3β levels did not significantly differ between fetal, neonatal, and adult HSCs/MPPs. Fetal HSCs/MPPs in G0/G1 versus S/G2/M phases of the cell cycle exhibited similar levels of AKT phosphorylation (Figure 1E).

We next evaluated AKT and S6 phosphorylation in sorted HSCs/MPPs from the bone marrow of untreated or cyclophosphamide/G-CSF-treated 8-week-old adult mice. After cyclophosphamide and 2 days of G-CSF, HSCs/MPPs did not exhibit increased AKT or S6 phosphorylation (Figure 1F) despite the high percentage of cells in S/G2/M (Figures 1B and 1C). Granulocyte-monocyte progenitors (Lineage$^-$c-kit$^+$Sca-1$^-$CD34$^+$CD16/32$^+$ cells; GMPs) from the same mice did have higher levels of phosphorylated AKT and S6 after cyclophosphamide/G-CSF treatment (Figure 1F). CD48$^-$LSK cells were also harvested from the spleens of mice treated with cyclophosphamide and 4 days of G-CSF. HSCs/MPPs from these mice still did not exhibit increased AKT phosphorylation relative to normal adult HSCs, though an increase in 4EBP phosphorylation was observed (Figure 1G). Rapidly dividing HSCs therefore exhibit little or no increase in AKT phosphorylation relative to quiescent adult HSCs.

Pten Is Required by Adult, but Not Neonatal, HSCs

To determine whether *Pten* regulates neonatal HSCs, we induced Cre in *Mx1-Cre; Pten*$^{fl/fl}$ mice by administering a single subcutaneous dose of pIpC 2 days after birth, resulting in complete *Pten* deletion in HSCs (Figure S1 available online). Bromo-deoxyuridine (BrdU) was then administered for 24 hr before the mice were sacrificed (Figures 2A and 2B). The overall proliferation of unfractionated bone marrow cells at 2, 3, 4, and 5 weeks after birth was not affected by *Pten* deletion (Figure 2A). The overall rate of BrdU incorporation into control CD150$^+$CD48$^-$Lin$^-$Sca-1$^+$c-Kit$^+$ HSCs declined from 2 (25% ± 8.5%) to 5 (10% ± 6.8%) weeks after birth as HSCs made the transition to a more slowly dividing adult phenotype (Figure 2B). *Pten* deletion had no effect on BrdU incorporation into HSCs at 2 weeks after birth, but *Pten*-deficient HSCs showed a trend toward increased BrdU incorporation relative to control HSCs at 3 weeks after birth, and the effect was statistically significant at 4 and 5 weeks after birth (Figure 2B).

To determine whether *Pten* deletion mobilizes neonatal HSCs, we administered pIpC to 2-day-old neonatal mice or to 6-week-old adult mice, then measured the number of HSCs per spleen at 2, 4, and 8 weeks after birth (Figure 2C). We observed a >40-fold increase in the number of HSCs in the spleens of 8-week-old adult mice (2 weeks after *Pten* deletion; Figure 2C). In contrast, *Pten* deletion had no effect on the number of HSCs in the spleen of 2-week-old mice and only modestly (but significantly) increased the number of HSCs in the spleens of 4-week-old mice (Figure 2C). We also did not observe any effect of *Pten* deletion on the overall proliferation of splenocytes until 4 weeks after birth (Figure 2D). *Pten* deletion therefore does not lead to increased HSC proliferation or mobilization until around 4 weeks after birth.

We compared the reconstituting capacity of HSCs from neonatal and adult mice after *Pten* deletion at 2 days or 6 weeks after birth. We transplanted 10 CD45.2$^+$ CD150$^+$CD48$^-$Lin$^-$Sca-1$^+$c-kit$^+$ HSCs from donor mice of each age and genotype into irradiated CD45.1$^+$ mice along with 300,000 wild-type CD45.1$^+$ bone marrow cells. Control adult (Figures 2E and 2F) and neonatal (Figures 2G and 2H) HSCs gave long-term multilineage reconstitution by myeloid and lymphoid cells in all recipient mice. *Pten*-deficient adult HSCs gave only transient multilineage reconstitution that lasted less than 16 weeks after transplantation in all recipient mice (Figures 2E and 2F). *Pten*-deficient neonatal HSCs gave long-term multilineage reconstitution in most recipients that lasted at least 16 weeks after transplantation, but none of these recipients were multilineage reconstituted after 24 weeks (Figures 2G and 2H). *Pten*-deficient neonatal HSCs therefore retained the capacity to give long-term multilineage reconstitution, suggesting that they did not initially depend upon *Pten*. The ultimate depletion of *Pten*-deficient neonatal HSCs 16 to 24 weeks after transplantation presumably reflects their maturation into adult HSCs as fetal HSCs acquire an adult phenotype after transplantation into adult mice (Figures S1B and S1C).

PTEN Prevents Leukemia in Adult, but Not Neonatal, Mice

To test whether *Pten* deletion causes neoplastic proliferation in neonatal mice, we treated littermate control and *Mx1-Cre; Pten*$^{fl/fl}$ mice with pIpC at 2 days or 6 weeks after birth. Two weeks after pIpC treatment was performed, we observed a 3-fold increase in spleens' cellularity and weight in adult, but not neonatal, *Pten*-deleted mice (Figures 2I and 2J). We also observed histological signs of myeloproliferative disorder in adult, but not neonatal, spleen after *Pten* deletion. Two weeks after *Pten* deletion in adult mice, spleens exhibited a substantial expansion of the red pulp and extramedullary hematopoiesis consistent with myeloproliferative disorder (Figures 2K and 2L). Two weeks after *Pten* deletion in neonatal mice, spleens had qualitatively fewer follicles than spleens from littermate controls (data not shown), consistent with the reduction in B cells in neonatal *Pten*-deficient mice (Figures 4J and 4K). Otherwise, the histological appearance of neonatal *Pten*-deficient and control spleens was indistinguishable (Figures 2M and 2N). *Pten* is therefore not required to prevent myeloproliferative disorder in neonatal mice.

When *Pten* is deleted from young adult mice they succumb to T-ALL by 12 weeks after Cre induction (Yilmaz et al., 2006; Lee et al., 2010). This latency period makes it difficult to test whether *Pten* deletion is leukemogenic in neonatal mice because they would mature into adults before the onset of leukemia; therefore, to test whether *Pten* is a tumor suppressor in neonatal mice, we

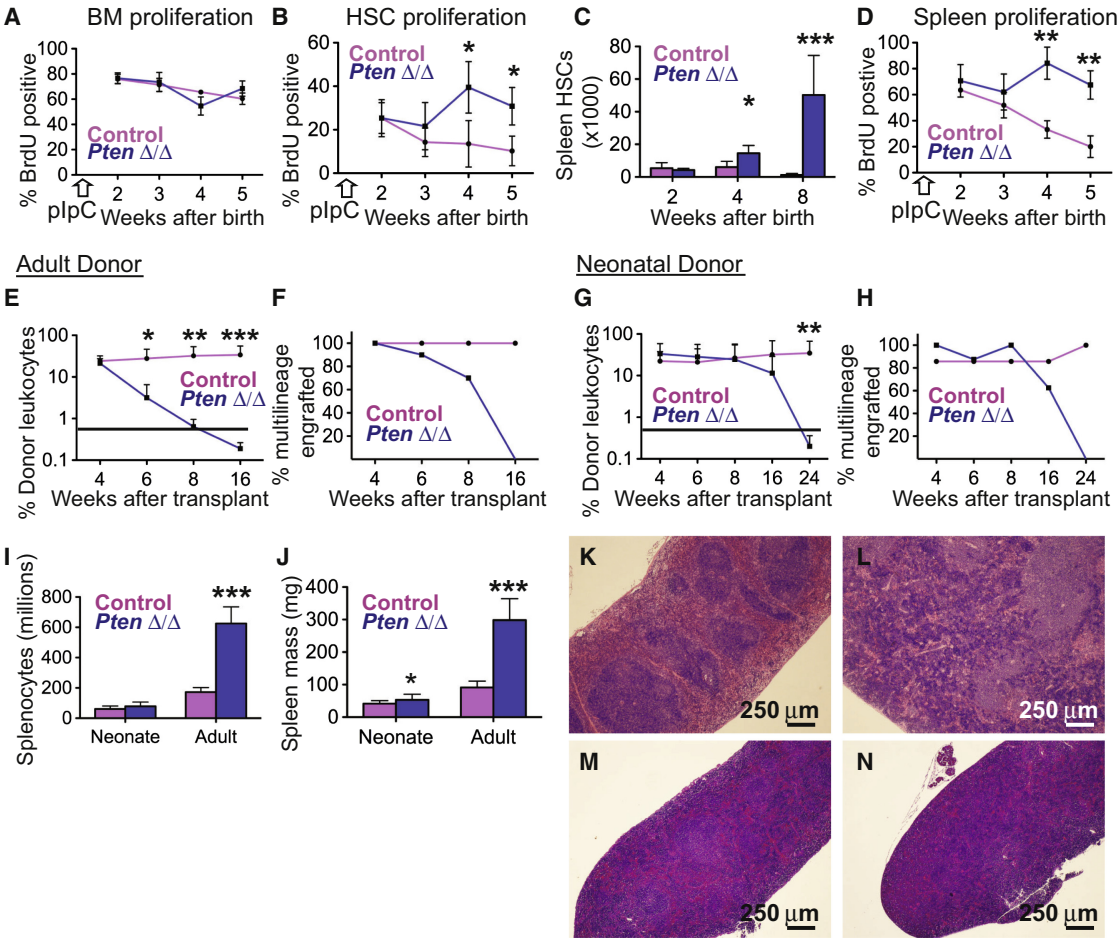

Figure 2. *Pten* Is Not Required to Regulate HSC Maintenance, Proliferation, or Function in Neonatal Mice

(A and B) BrdU incorporation in bone marrow cells and HSCs (24 hr pulse; three or four independent experiments; *p < 0.05).

(C) The numbers of HSCs in the spleens of mice that received pIpC 2 days after birth then were then analyzed at 2 or 4 weeks after birth, or those that received pIpC at 6 weeks after birth were then analyzed 8 weeks after birth (n = 4–10 mice per genotype, *p < 0.05; ***p < 0.0001).

(D) Splenocyte BrdU incorporation (24 hr BrdU pulse; n = 3–4 mice per time point and genotype; **p < 0.01).

(E–H) Long-term competitive repopulation assays with 10 wild-type or *Pten*-deficient (*Pten* Δ/Δ) adult or neonatal HSCs (n = 7–10 recipients/age/genotype; *p < 0.05; **p < 0.01; ***p < 0.001). The threshold at which donor cells could be detected above background (in negative control mice, 0.5%) is indicated by the black line.

(I and J) Spleen cellularity (I) and mass (J) for *Pten*-deleted adult and neonatal *Mx1-Cre; Pten*$^{fl/fl}$ mice as well as littermate controls 2 weeks after pIpC treatment (n = 10–13 mice/age/genotype; *p < 0.05; ***p < 1 × 10^{-10}).

(K–N) Representative low power (40×) spleen sections stained with H&E from wild-type adult (K), *Pten*-deleted adult (L), wild-type neonatal (M), and *Pten*-deleted neonatal (N) mice.

For all panels, error bars reflect standard deviation and p values were calculated by two-tailed Student's t test.

deleted *Pten* from *p53*-deficient mice. In adult mice this greatly accelerates leukemogenesis (Lee et al., 2010). This allowed us to test whether neonatal mice are immediately competent to develop leukemia after *Pten* deletion or whether an adult developmental context is required. We administered pIpC to *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice and littermate controls (that lacked *Mx1-Cre*) at 2 days or 6 weeks after birth. By 20 days after pIpC treatment, all adult *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice died with signs of leukemia (Figure 3A). Control *p53*$^{-/-}$ or *p53*$^{+/-}$ adult mice that retained *Pten* all survived for at least 100 days with no evidence of hematopoietic neoplasms (Figures 3A and 3B). All moribund adult *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice had large mediastinal masses and infiltrative precursor T-ALL (Figures 3E–3H). Adult *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice therefore developed T-ALL and died within 20 days of pIpC.

At 21 days after pIpC treatment, when all of the adult *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice were dead, none of the neonatal *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice that had been treated with pIpC at 2 days of age showed any signs of illness (Figure 3A). These mice did not die until 44 to 60 days after pIpC treatment, when they were 6- to 8-week-old adults (Figure 3A). Indeed, the *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice died at the same time after birth irrespective of whether pIpC was administered at 2 days or 6 weeks after birth (Figure 3C). When neonatal *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice were sacrificed at 17 days after pIpC treatment (a time point at which all adult *Mx1-Cre; Pten*$^{fl/fl}$*; p53*$^{-/-}$ mice showed clear signs

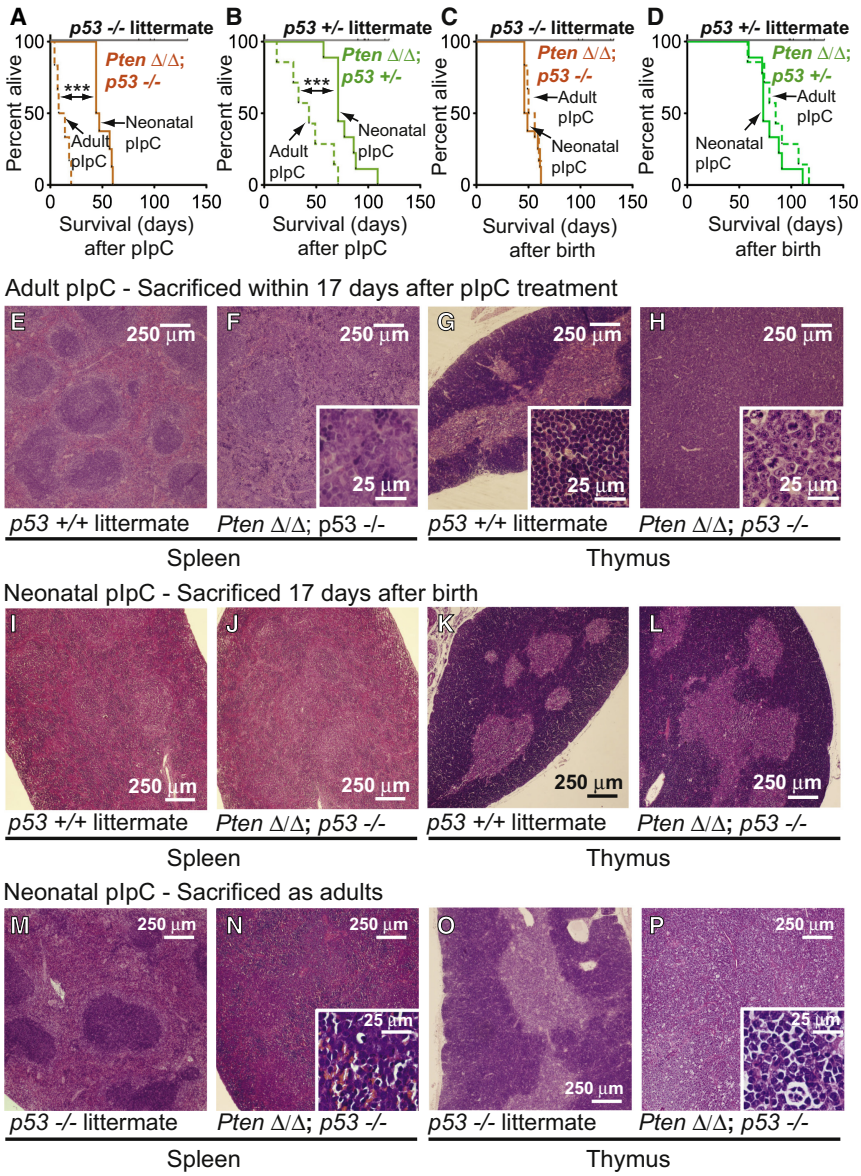

Figure 3. Loss of *Pten* and *p53* Leads to the Development of T-ALL in Adult, but Not Neonatal, Mice

(A–D) Kaplan-Meier survival curves for *Mx1-Cre; Pten$^{fl/fl}$; p53$^{-/-}$* (A and C; n = 6–8 mice/treatment) and *Mx1-Cre; Pten$^{fl/fl}$; p53$^{+/-}$* mice (B and D; n = 7–9 mice/treatment) following pIpC treatment at 2 days (neonatal) or 6 weeks (adult) after birth. Survival times are shown either as the time following pIpC treatment (A and B) or as time after birth (C and D). *Pten* mutant mice that were treated neonatally with pIpC survived significantly longer after pIpC treatment than mice treated with pIpC as adults (***$p < 0.001$ by the log rank test).

(E–H) Spleen and thymus sections from adult mice that were treated with pIpC at 6 weeks after birth and sacrificed for analysis 17 days later (40×, inset 400×).

(I–L) Spleen and thymus sections from neonatal mice treated with pIpC 2 days after birth and sacrificed for analysis 17 days later (40×).

(M–P) Spleen and thymus sections from mice that received pIpC 2 days after birth and developed T-ALL as adults (40×, inset 400×).

of T-ALL), none of the neonatally treated mice showed any signs of illness or leukemia: they had normal thymus and spleen histology (Figures 3I–3L); however, when these mice became ill approximately 50 days after pIpC treatment, they had large mediastinal masses and infiltrative T-ALL (Figures 3M–3P). The observation that *Mx1-Cre; Pten$^{fl/fl}$; p53$^{-/-}$* mice succumbed to leukemia at the same time, irrespective of whether *Pten* was deleted 2 days or 6 weeks after birth (Figure 3C), demonstrates that an adult developmental context is required for leukemia after *Pten* deletion. We performed similar experiments in mice with a *p53* heterozygous background and obtained similar results (Figures 3B and 3D).

Pten Deletion Increases PI3-Kinase Pathway Activation in Adult, but Not Neonatal, HSCs

To test whether *Pten* is required in neonatal HSCs to regulate PI3-kinase pathway signaling, we administered pIpC to *Mx1-Cre; Pten$^{fl/fl}$* mice or littermate controls at 2 days or 6 weeks after birth. We isolated CD48^{-}LSK cells 2 weeks later and performed western blots. The total levels of PTEN, AKT, S6, and GSK3β were similar in neonatal and adult HSCs/MPPs from control mice (Figures 4A and 4B). In both the neonatal and adult HSCs/MPPs, we observed a complete loss of PTEN protein from *Mx1-Cre; Pten$^{fl/fl}$* cells (Figures 4A and 4B). In adult HSCs/MPPs, *Pten* deletion increased the phosphorylation of AKT, S6, and GSK3β, but not MAPK or AMPK (Figures 4A and 4B). In neonatal HSCs/MPPs, *Pten* deletion had little or no affect on AKT, S6, and GSK3β phosphorylation (Figures 4A and 4B). *Pten* deletion did not increase AKT phosphorylation in HSCs/MPPs until 3 to 4 weeks after birth (Figure 4C), the same time we began to observe increased HSC proliferation after *Pten* deletion (Figure 2B).

To determine whether neonatal HSCs/MPPs are capable of activating the PI3-kinase pathway, we incubated neonatal or adult HSCs/MPPs from control and *Mx1-Cre; Pten$^{fl/fl}$* mice in culture with 2% fetal bovine serum at 37°C for 30 min. In contrast to uncultured neonatal *Pten*-deficient HSCs/MPPs, phosphorylated AKT was present at comparable levels in neonatal versus adult *Pten*-deficient HSCs/MPPs (Figure 4D). Neonatal HSCs are therefore capable of phosphorylating AKT when exposed to serum in culture and PTEN negatively regulates this process. Several PI3K pathway proteins, including AKT, S6, RICTOR, mTOR, RAPTOR, and SHIP1, are expressed at similar levels in neonatal and adult HSCs (Figures 4A, 4B, and 4E). Phosphorylation of mTOR at Ser2481, which reflects mTORC2 function (Copp et al., 2009), was also similar in neonatal and adult

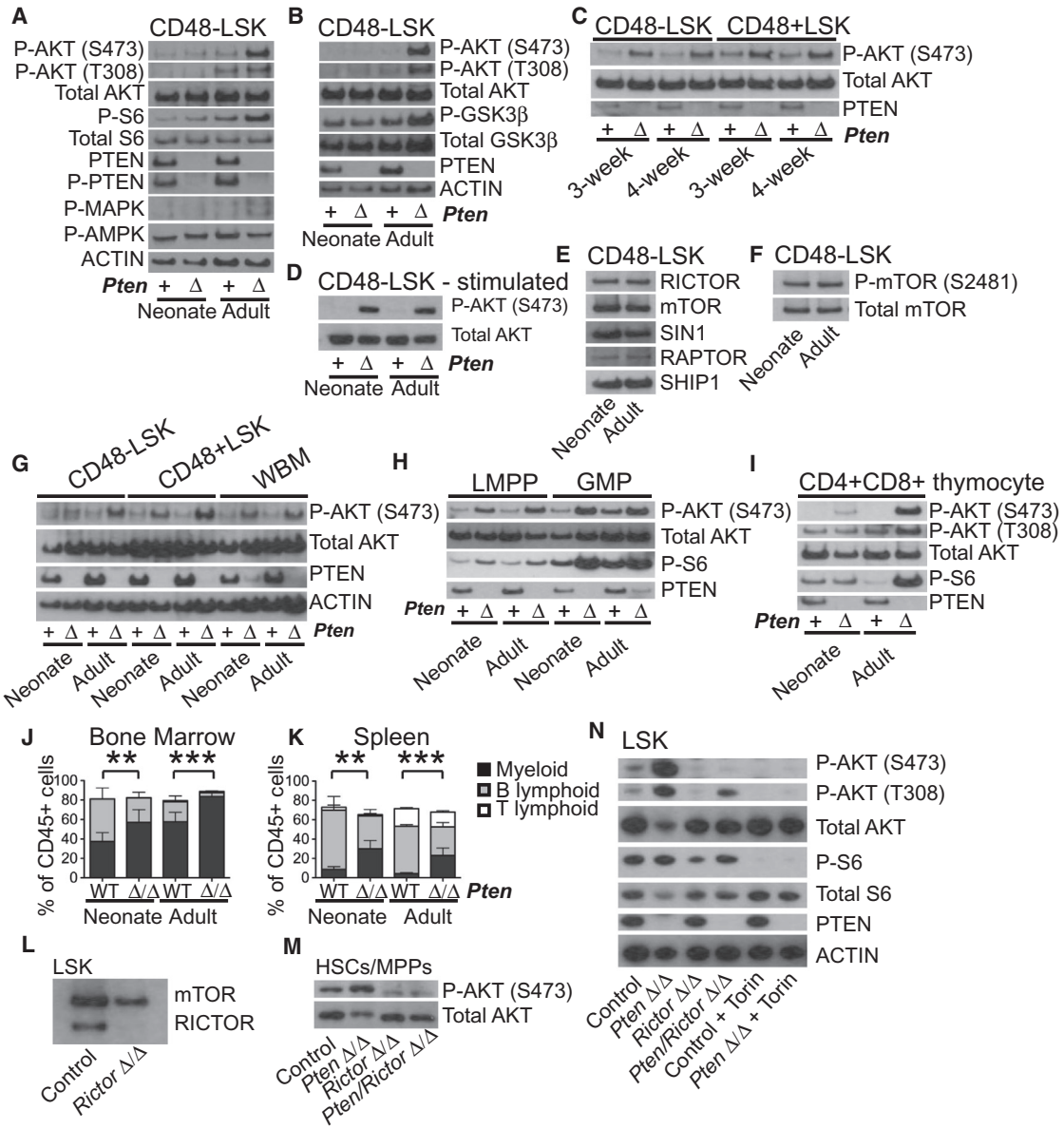

Figure 4. Neonatal HSCs Do Not Require PTEN to Negatively Regulate AKT Phosphorylation by mTORC2 In Vivo

(A) Western blots were performed on 30,000 wild-type or *Pten*-deficient CD48$^-$LSK cells isolated from adult and neonatal mice. After *Pten* deletion AKT and S6 phosphorylation increased in adult HSCs/MPPs but little increase was observed in neonatal HSCs/MPPs.

(B) AKT and GSK3β phosphorylation increased in adult, but not neonatal, HSCs/MPPs after *Pten* deletion.

(C) AKT phosphorylation in 20,000 HSCs/MPPs from 3- and 4-week-old control and *Pten*-deficient mice.

(D) HSCs/MPPs from adult and neonatal mice were incubated in Iscove's medium with 2% fetal bovine serum for 30 min.

(E) The expression levels of several PI3-kinase pathway components were similar in adult and neonatal HSCs/MPPs (30,000 cells per lane).

(F) Phosphorylation of mTOR at Ser2481 was not different between adult and neonatal HSCs/MPPs.

(G) AKT phosphorylation in 20,000 wild-type or *Pten*-deficient CD48$^-$LSK cells (HSCs/MPPs), CD48$^+$LSK cells (restricted progenitors), or bone marrow cells from adult or neonatal mice.

(H and I) AKT and S6 phosphorylation in neonatal and adult, control and *Pten*-deficient LMPPs (H), GMPs (H), and CD4+CD8+ thymocytes (I).

(J and K) In both adult and neonatal mice, *Pten* deletion significantly reduced B cell frequencies and significantly increased myeloid cell frequencies (n = 6–7 mice/age/genotype; $^{**}p < 0.01$; $^{***}p < 0.001$). Error bars always represent standard deviation.

(L) pIpC treatment of *Mx1-Cre; Rictor$^{fl/fl}$* mice eliminated RICTOR expression by LSK cells.

(M and N) *Rictor* deletion reduced AKT Ser473 phosphorylation in CD48$^-$LSK cells (M), and had similar effects to Torin1 treatment with respect to AKT phosphorylation at Ser473, but not with respect to S6 phosphorylation (N).

HSCs/MPPs (Figure 4F). A number of other gene products known to regulate the PI3-kinase pathway were expressed at similar levels in neonatal and adult HSCs by quantitative RT-PCR (Figure S2A). Neonatal HSCs/MPPs are therefore capable of activating the PI3-kinase pathway, but PTEN is not required in neonatal HSCs/MPPs in vivo to negatively regulate pathway activation.

Neonatal restricted progenitors ($CD48^+$LSK cells) and unfractionated bone marrow cells did exhibit increased AKT phosphorylation following *Pten* deletion (Figure 4G). *Pten* deletion also increased AKT and S6 phosphorylation in lymphoid primed multipotent progenitors ($Lineage^-c\text{-}kit^+Sca\text{-}1^+CD34^+FLT3^+$ cells; LMPPs) and GMPs from neonatal and adult mice (Figure 4H). In contrast, adult $CD4^+CD8^+$ thymocytes had much higher levels of AKT and S6 phosphorylation after *Pten* deletion than neonatal $CD4^+CD8^+$ thymocytes (Figure 4I). As in prior studies (Yilmaz et al., 2006; Zhang et al., 2006), we observed an increase in myelopoiesis and a decrease in B lymphopoiesis in adult bone marrow after *Pten* deletion. We also observed an increase in myelopoiesis and a decrease in B lymphopoiesis in the bone marrow and spleen of neonatal mice after *Pten* deletion (Figures 4J and 4K). These data demonstrate that many restricted hematopoietic progenitors in neonatal mice depend upon PTEN to regulate cellular signaling and function, just as observed in adult mice, but that HSCs and T lineage progenitors undergo a temporal change in their dependence upon PTEN between the neonatal and adult stages.

To further explore the differences in PI3-kinase pathway regulation between neonatal and adult HSCs, we compared the gene expression profiles of P14 neonatal HSCs and 8-week-old adult HSCs from wild-type mice. We identified 14 genes that were significantly more highly expressed in neonatal HSCs, and 21 genes that were significantly more highly expressed in adult HSCs (Table S2; fold change >2.0). Ten of these genes could potentially regulate PI3-kinase pathway signaling based on prior studies. The differential expression of eight of these genes was confirmed by quantitative RT-PCR (Figure S2B, Table S3). For example, *Bank1* was expressed more highly in neonatal relative to adult HSCs (Figure S2B) and is known to negatively regulate AKT phosphorylation (Aiba et al., 2006). This provides candidate genes that could potentially explain the difference in PTEN dependence between neonatal and adult HSCs, but gene-targeted mice will have to be generated to functionally test these hypotheses.

Rictor Deletion Reduces AKT Phosphorylation in Primitive Hematopoietic Progenitors

We next assessed the mechanisms by which *Pten* deletion promotes HSC depletion and leukemogenesis in adult mice. *Pten* deletion can activate both mTORC1 and mTORC2 (Guertin and Sabatini, 2007); however, mTORC1 promotes proliferation by phosphorylating S6 kinase and 4EBP1 while mTORC2 phosphorylates AKT at Ser473 (Guertin et al., 2006; Guertin and Sabatini, 2007). Since increased AKT phosphorylation at Ser473 distinguished *Pten*-deficient HSCs from HSCs that were dividing under physiological conditions (Figure 1C), we tested whether the effects of *Pten* deficiency were mediated by increased mTORC2 activation. We generated a floxed allele that allowed us to conditionally delete *Rictor* (Figure S3), a key component of the mTORC2 complex (Guertin et al., 2006), and we generated *Mx1-Cre; Rictor$^{fl/fl}$* mice. We administered pIpC 6 weeks after birth and observed an almost complete loss of RICTOR protein from *Mx1-Cre; Rictor$^{fl/fl}$* LSK cells, but mTOR was still expressed (Figure 4L). We confirmed that both *Rictor* alleles were recombined in all HSCs tested (Figure S4A).

To determine whether *Rictor* deletion attenuates AKT phosphorylation in HSCs/MPPs, we administered pIpC to control, *Mx1-Cre; Pten$^{fl/fl}$*, *Mx1-Cre; Rictor$^{fl/fl}$*, and *Mx1-Cre; Pten$^{fl/fl}$; Rictor$^{fl/fl}$* mice 6 weeks after birth. As expected, *Pten* deletion increased AKT phosphorylation at Ser473 (Figure 4M). *Rictor* deletion substantially reduced AKT Ser473 phosphorylation in HSCs/MPPs, to levels lower than observed in control HSCs/MPPs, even when *Pten* and *Rictor* were simultaneously deleted (Figure 4M). This result is consistent with the expectation that *Rictor* deletion reduces mTORC2 function.

To further evaluate RICTOR regulation of the PI3-kinase pathway, we harvested LSK cells from 8-week-old control, *Mx1-Cre; Pten$^{fl/fl}$*, *Mx1-Cre; Rictor$^{fl/fl}$*, and *Mx1-Cre; Pten$^{fl/fl}$; Rictor$^{fl/fl}$* mice, 2 weeks after pIpC treatment. These cells were incubated for 30 min in Iscove's medium + 2% fetal bovine serum before being harvested for western blotting. In parallel, control and *Pten*-deficient LSK cells were incubated with 250 nM Torin1, an active site inhibitor of mTOR that inhibits both mTORC1 and mTORC2 kinase activity (Thoreen et al., 2009). *Pten* deletion increased AKT phosphorylation, and *Rictor* deletion or Torin1 treatment eliminated AKT phosphorylation, both in wild-type and *Pten*-deficient LSK cells (Figure 4N). We also observed reduced AKT phosphorylation at Thr308 in *Mx1-Cre; Pten$^{fl/fl}$; Rictor$^{fl/fl}$* LSK cells relative to *Mx1-Cre; Pten$^{fl/fl}$* LSK cells, and Torin1 treatment completely blocked AKT phosphorylation at Thr308 even though this site is not thought to be an mTORC1 or mTORC2 substrate (Figure 4N). A similar decrease in Thr308 phosphorylation was previously observed in *Rictor*-deficient prostate epithelium (Guertin et al., 2009). These results confirm that *Rictor* deletion reduces mTORC2 function and AKT phosphorylation in primitive hematopoietic progenitors.

Rictor Is Not Necessary for HSC Maintenance in Neonatal or Adult Mice

To test if RICTOR is required by hematopoietic cells, we analyzed *Vav-Cre; Rictor$^{fl/fl}$* mice and *Mx1-Cre; Rictor$^{fl/fl}$* mice. *Vav-Cre* expresses in HSCs during early embryonic development (de Boer et al., 2003). *Vav-Cre; Rictor$^{fl/fl}$* mice were born at Mendelian ratios and they matured to adulthood without any gross abnormalities (data not shown). We did not observe significant differences in bone marrow cellularity, spleen cellularity, spleen weight, or thymus weight between *Vav-Cre; Rictor$^{fl/fl}$* neonatal mice and littermate controls 14 days after birth (Figures S4C and S4D). The frequency and absolute number of HSCs in the bone marrow of *Vav-Cre; Rictor$^{fl/fl}$* neonatal mice was slightly but significantly elevated relative to littermate controls (Figure 5A; Figure S4E). We also deleted *Rictor* 2 days after birth by administering a single dose of pIpC to *Mx1-Cre; Rictor$^{fl/fl}$* mice to achieve complete recombination of the *Rictor^{fl}* alleles (Figure S4B). At P14 we did not observe significant differences in the frequency or absolute number of bone marrow HSCs between *Mx1-Cre; Rictor$^{fl/fl}$* mice and littermate controls

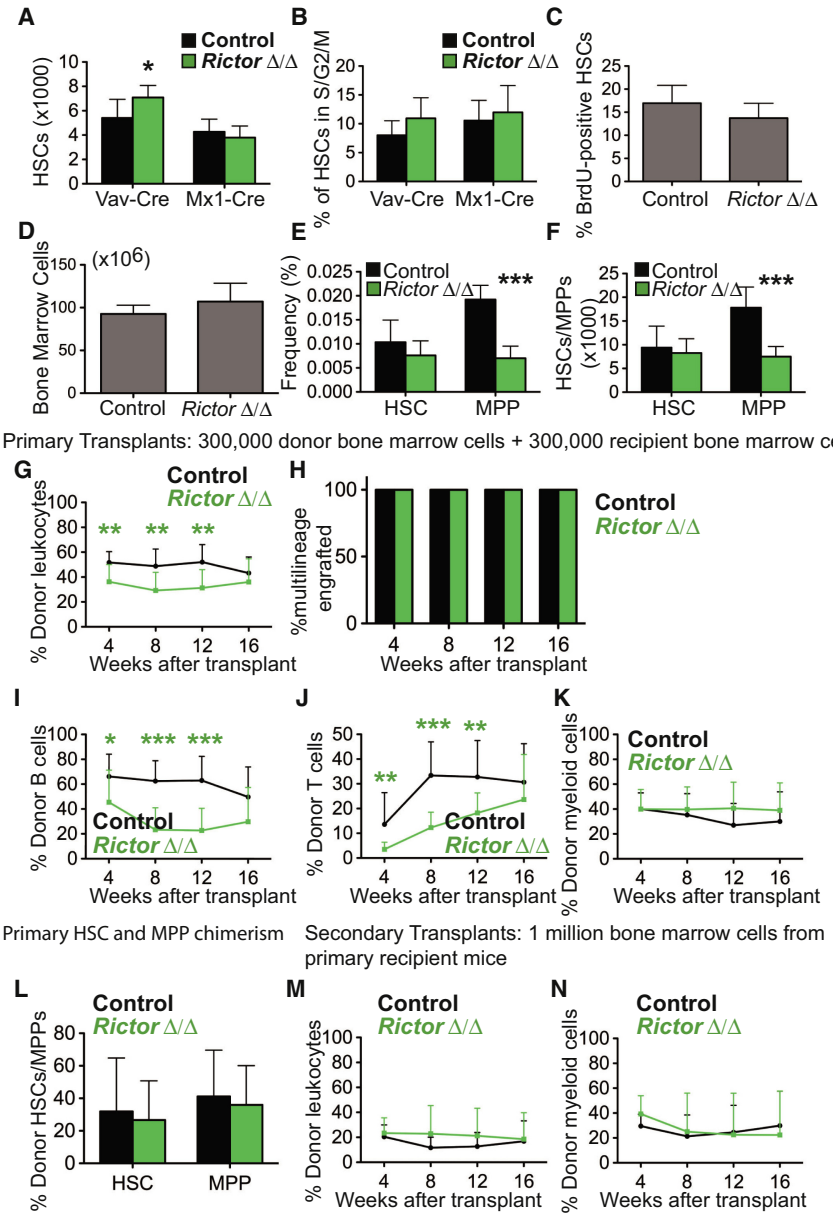

Figure 5. *Rictor* Deletion Had Only Modest Effects on HSC Frequency and Function

(A) *Vav-Cre*-mediated *Rictor* deletion in fetal HSCs slightly but significantly increased HSC numbers in P14 mice (n = 5–7 mice per genotype, *p < 0.05). *Mx1-Cre*-mediated *Rictor* deletion at P2 did not significantly affect HSC numbers at P14.

(B and C) *Vav-Cre* or neonatal *Mx1-Cre*-mediated *Rictor* deletion did not affect cell cycle distribution (B) or BrdU incorporation (C) in HSCs at P14.

(D–F) Deletion of *Rictor* in 6-week-old adult *Mx1-Cre;Rictor*$^{fl/fl}$ mice did not significantly affect bone marrow cellularity (D), HSC frequency (E), or absolute HSC number (F) when analyzed 18–24 weeks after pIpC treatment. However, MPP frequency (E) and MPP numbers (F) were reduced in *Rictor*-deleted mice relative to control mice (n = 5–7 mice per genotype, ***p < 0.001).

(G–K) When 300,000 control or *Rictor*-deficient CD45.2 bone marrow cells were transplanted along with 300,000 CD45.1 wild-type bone marrow cells into irradiated mice, recipients of *Rictor*-deficient bone marrow were always long-term multilineage reconstituted by donor cells but levels of donor cell reconstitution were sometimes significantly lower than in recipients of control cells (n = 11–14 recipients per genotype, *p < 0.05; **p < 0.01; ***p < 0.001).

(L) The frequencies of donor HSCs and MPPs were similar in primary recipients of control and *Rictor*-deficient bone marrow cells (n = 11–14 per genotype).

(M and N) Secondary recipients of *Rictor*-deficient and control bone marrow cells exhibited similar levels of long-term multilineage reconstitution (n = 15 recipients per genotype).

Error bars reflect standard deviation and p values were calculated by two-tailed Student's t tests.

(Figure 5A; Figure S4E). *Rictor* is therefore not necessary for the formation of HSCs or for fetal/neonatal hematopoiesis.

To determine whether *Rictor* regulates the proliferation of neonatal HSCs, we evaluated the cell cycle status of HSCs isolated at P14 from *Vav-Cre; Rictor*$^{fl/fl}$ mice and *Mx1-Cre; Rictor*$^{fl/fl}$ mice that received pIpC at P2. We did not observe significant differences between control and *Rictor*-deficient HSCs in the frequency of HSCs in S/G2/M phases of the cell cycle in either genetic background (Figure 5B). We also observed no significant difference in the BrdU incorporation rate of *Rictor*-deficient and control HSCs (Figure 5C). *Rictor* is therefore not required to regulate the maintenance or cell cycle of neonatal HSCs.

To determine whether *Rictor* regulates adult hematopoiesis or HSC function, we administered pIpC to adult *Mx1-Cre; Rictor*$^{fl/fl}$ mice and littermate controls 6 weeks after birth. These mice were observed for 18–24 weeks following pIpC treatment and they did not develop any overt signs of illness (data not shown). After the observation period, we detected a small, but significant, decrease in white blood cell counts in *Rictor*-deleted mice (Figure S4F), but red blood cell counts (Figure S4G), platelet counts (Figure S4H), and thymus cellularity (Figure S4I) were not significantly affected by *Rictor* deletion. We observed a trend toward reduced spleen cellularity in multiple experiments but the effects were not statistically significant (Figure S4L). We also observed no significant difference in bone marrow cellularity, HSC frequency, or absolute HSC numbers (Figures 5D–5F). We did observe a significant reduction in the frequency of MPPs ($CD150^{-}CD48^{-}$LSK) after *Rictor* deletion (Figures 5E and 5F). *Rictor* is therefore not required for the maintenance of adult HSCs, but RICTOR may regulate MPPs.

Rictor-deficient bone marrow cells gave long-term multilineage reconstitution of all recipient mice for at least 16 weeks after transplantation (Figures 5G–5K). We observed significantly

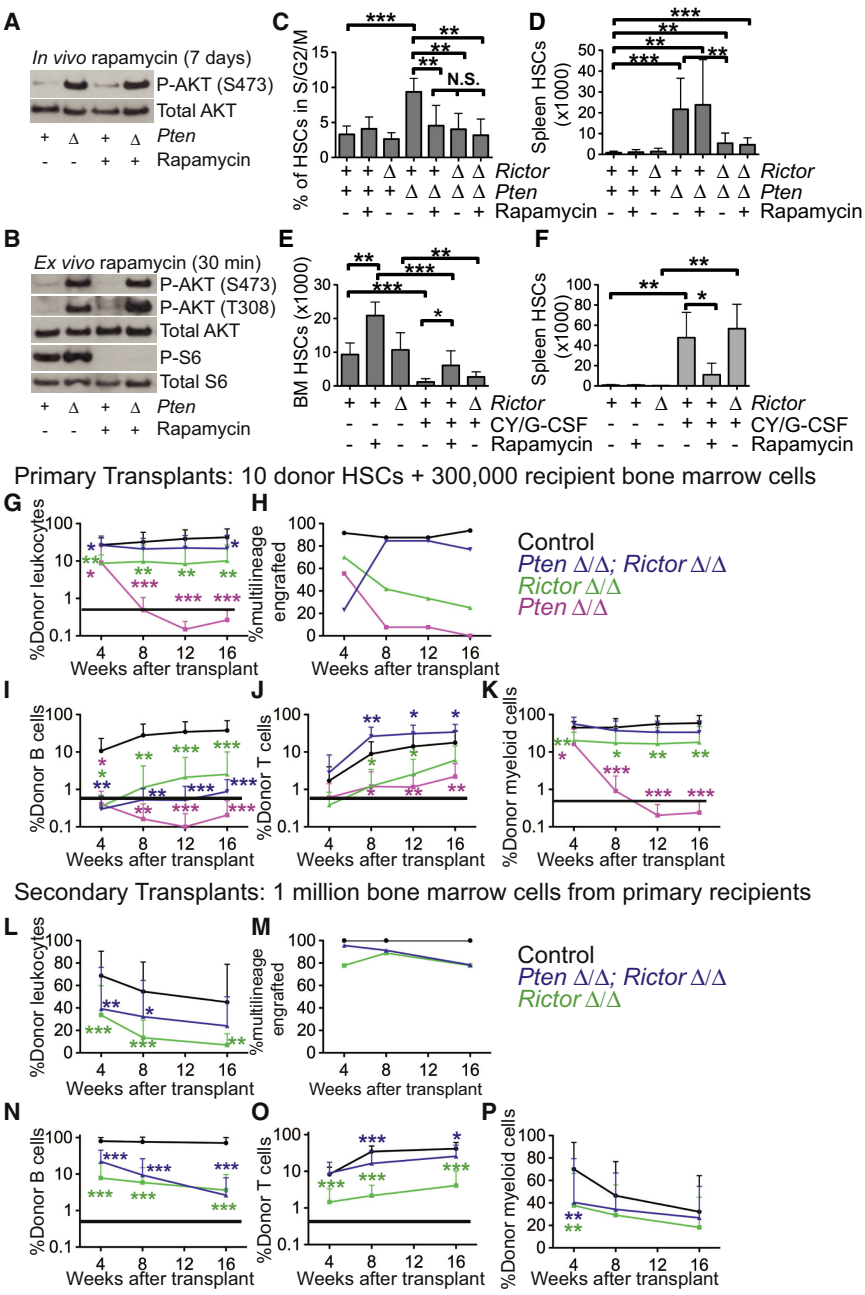

Figure 6. *Rictor* Is Necessary for Increased HSC Proliferation, HSC Mobilization, and HSC Depletion following *Pten* Deletion

(A and B) Rapamycin did not inhibit AKT phosphorylation after 7 days in vivo (A) or 30 min in vitro (B).

(C) The percentage of HSCs in S/G2/M phases of the cell cycle in control, *Mx1-Cre; Rictor*$^{fl/fl}$ (*Rictor*Δ/Δ), *Mx1-Cre; Pten*$^{fl/fl}$ (*Pten*Δ/Δ), and *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ (*Pten*Δ/Δ; *Rictor*Δ/Δ) mice, with or without 7 days of rapamycin treatment, 2 weeks after pIpC treatment (n = 3–10 mice per genotype and treatment).

(D) *Rictor* deletion, but not rapamycin treatment, significantly reduced HSC mobilization to the spleen following *Pten* deletion (n = 8–9 mice/genotype).

(E and F) Rapamycin treatment, but not *Rictor* deletion, increased bone marrow HSC numbers (E) and reduced HSC mobilization to the spleen following cyclophosphamide/G-CSF treatment (F; n = 5 mice/treatment).

(G–K) Ten CD45.2+ donor HSCs from *Pten*-deficient mice failed to give long-term multilineage reconstitution in irradiated mice. *Rictor*-deficient HSCs gave long-term multilineage reconstitution in 3 of 12 recipient mice, but levels of donor cell reconstitution were generally lower than observed from control HSCs. *Pten*; *Rictor* compound-deficient HSCs were able to give long-term multilineage reconstitution in nearly all irradiated mice at levels similar to those of control HSCs, with the exception of the B cell lineage (I) (n = 12–16 mice/genotype).

(L–P) Following secondary transplantation, *Rictor*-deficient and *Pten*; *Rictor* compound-deficient donor cells gave long-term multilineage reconstitution of nearly all secondary recipient mice (n = 9–22 mice/genotype).

Error bars represent standard deviation and p values were calculated by two-tailed Student's t tests: *p < 0.05; **p < 0.01; ***p < 0.001; N.S., not significant (p > 0.05).

reduced levels of donor lymphocyte reconstitution in recipients of *Rictor*-deficient bone marrow cells over the first 12 weeks after transplantation, but this difference was not significant by 16 weeks after transplantation (Figures 5I and 5J). We did not observe any differences in the frequency of donor HSCs or MPPs in recipients of *Rictor*-deficient or control cells 16 weeks after transplantation (Figure 5L). We also did not observe significant differences in the reconstituting capacity of *Rictor*-deficient and control bone marrow cells in secondary recipients (Figures 5M and 5N). *Rictor* is therefore not necessary for HSC maintenance or function in normal adult mice, though its absence may lead to modest deficits in lymphoid reconstitution.

Rictor Is Necessary for Adult HSC Proliferation and Depletion after *Pten* Deletion

We administered pIpC to control, *Mx1-Cre; Pten*$^{fl/fl}$, *Mx1-Cre; Rictor*$^{fl/fl}$, and *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice 6 weeks after birth and analyzed HSCs 2 weeks later. Since rapamycin rescues the proliferation of *Pten*-deficient HSCs (Yilmaz et al., 2006), we also administered rapamycin to some *Mx1-Cre; Pten*$^{fl/fl}$ mice and controls for 1 week prior to analysis. Rapamycin did not appear to affect mTORC2 function in primitive hematopoietic progenitors as it did not affect AKT phosphorylation at Ser473 in *Pten*-deficient LSK cells in vivo (7 days at 4 mg/kg/day; Figure 6A), or after 30 min of culture in rapamycin-containing medium (100 nm; Figure 6B). In contrast, *Rictor* deletion may modestly reduce mTORC1 activation as phosphorylated S6 levels were modestly reduced in *Rictor*-deficient as compared to control LSK cells and phosphorylated S6 levels were reduced as a fraction of total S6 levels in *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ LSK

cells relative to *Mx1-Cre; Pten*$^{fl/fl}$ LSK cells (Figure 4N). A decline in mTORC1 signaling after *Rictor* deletion may be expected as mTORC2 signaling increases AKT signaling and therefore mTORC1 activation (Guertin and Sabatini, 2007; Guertin et al., 2009). In primitive hematopoietic progenitors rapamycin thus appears to reduce mTORC1 function without affecting mTORC2 while *Rictor* deletion appears to eliminate mTORC2 function while modestly reducing mTORC1 function.

Pten deletion significantly increased the fraction of HSCs in S/G2/M phases of the cell cycle and rapamycin rescued this effect (Figure 6C). *Rictor* deletion by itself did not significantly affect the frequency of HSCs in S/G2/M phases of the cell cycle but did significantly reduce the fraction of *Pten*-deficient HSCs in S/G2/M phases of the cell cycle (Figure 6C). Treating *Pten*/*Rictor* compound mutant mice with rapamycin did not further reduce HSC proliferation. *Rictor* deletion also significantly reduced the mobilization of *Pten*-deficient HSCs but rapamycin had no effect (Figure 6D). Taken together this suggests that mTORC2 mediates the effects of *Pten* deletion on HSC function through mTORC1-dependent and mTORC1-independent mechanisms, consistent with studies in other cell types (Guertin and Sabatini, 2007).

Rapamycin treatment, but not *Rictor* deletion, reduced the mobilization of wild-type HSCs from the bone marrow to the spleen after cyclophosphamide/G-CSF treatment (Figures 6E and 6F). This suggests that mTORC1, not mTORC2, is the major mediator of HSC proliferation under physiological conditions in wild-type mice. This conclusion is consistent with the paucity of Ser473 phosphorylation of AKT (an mTORC2 site; Guertin et al., 2006) in fetal, neonatal, and cyclophosphamide/G-CSF mobilized HSCs (Figures 1D–1G) and with the lack of effect of *Rictor* deletion on HSC frequency or proliferation (Figure 5). *Pten* deletion therefore induces the proliferation and mobilization of HSCs through mTORC2-dependent mechanisms that do not mimic physiological HSC self-renewal mechanisms.

To test if *Rictor* is necessary for the depletion of *Pten*-deficient HSCs, we administered pIpC to 6-week-old control, *Mx1-Cre; Pten*$^{fl/fl}$, *Mx1-Cre; Rictor*$^{fl/fl}$, and *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice, then isolated HSCs 2 weeks later. We transplanted 10 CD45.2$^+$ HSCs from donor mice into irradiated CD45.1$^+$ mice along with 300,000 wild-type CD45.1$^+$ bone marrow cells. As expected, control HSCs gave high levels of long-term multilineage reconstitution in all recipient mice and *Pten*-deficient HSCs gave only transient multilineage reconstitution in all recipient mice (Figures 6G–6K). *Rictor*-deficient HSCs gave long-term multilineage reconstitution in 3 of 12 recipient mice. Most of the remaining mice had long-term myeloid reconstitution, suggesting that HSC activity was maintained in these mice, but they did not necessarily have long-term lymphoid reconstitution (Figures 6G–6K). In contrast to *Pten*-deficient HSCs, *Pten; Rictor* compound mutant HSCs gave long-term multilineage reconstitution in almost all recipient mice with levels of donor myeloid and T cell reconstitution that were at least as high as from control HSCs (Figures 6G–6K). *Pten; Rictor* compound mutant HSCs gave much lower levels of B lineage reconstitution than control HSCs (Figure 6I), suggesting that *Rictor* deficiency largely rescued the function of *Pten*-deficient HSCs but did not rescue the function of *Pten*-deficient B lineage progenitors.

Secondary recipients of *Rictor*-deficient cells exhibited long-term multilineage reconstitution in most mice, but levels of donor cells were significantly lower than from control cells (Figures 6L–6P). Secondary recipients of *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ cells also exhibited long-term multilineage reconstitution in most recipient mice, with levels of donor cell reconstitution that were intermediate between control and *Rictor*-deficient cells (Figures 6L–6P). *Rictor* deficiency therefore substantially rescued HSC function after *Pten* deletion, enabling *Pten* deficient HSCs to give long-term multilineage reconstitution of primary and secondary recipient mice.

Rictor Is also Required for Leukemogenesis following *Pten* Deletion

To determine whether *Rictor* is necessary for the development of myeloproliferative disorder and leukemia following *Pten* deletion, we administered pIpC to control, *Mx1-Cre; Pten*$^{fl/fl}$, *Mx1-Cre; Rictor*$^{fl/fl}$, and *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice 6 weeks after birth. We administered rapamycin for 7 days to some of these mice. We harvested spleens 2 weeks after the initial pIpC treatment. Spleens from *Mx1-Cre; Pten*$^{fl/fl}$ mice were significantly more cellular than spleens of other genotypes, as expected (Figure 7A). Spleens from *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ and rapamycin-treated *Mx1-Cre; Pten*$^{fl/fl}$ mice were also significantly more cellular than control spleens, but they were significantly less cellular than spleens from *Mx1-Cre; Pten*$^{fl/fl}$ mice (Figure 7A). Rapamycin in *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice further reduced spleen cellularity, rendering it similar to control spleens (Figure 7A). *Rictor* deletion and rapamycin treatment therefore additively reduce the severity of myeloproliferative disorder after *Pten* deletion, suggesting that mTORC1 and mTORC2 both contribute to this phenotype (Figure 7A). Nonetheless, extramedullary hematopoiesis was still histologically evident in the spleens of rapamycin-treated *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice, suggesting that rapamycin treatment and *Rictor* deletion did not completely eliminate the myeloproliferative disorder (Figure S5).

To determine whether *Rictor* is necessary for progression to acute leukemia following *Pten* deletion, we administered pIpC to control, *Mx1-Cre; Pten*$^{fl/fl}$, *Mx1-Cre; Pten*$^{fl/+}$*; Rictor*$^{fl/fl}$, *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/+}$, and *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice 6 weeks after birth. All control and *Mx1-Cre; Pten*$^{fl/+}$*; Rictor*$^{fl/fl}$ mice survived for the 115 day duration of the experiment (n = 7–11 mice/treatment; Figure 7B) with no signs of leukemia (Figures 7F and 7J). All *Mx1-Cre; Pten*$^{fl/fl}$ mice died by 77 days after pIpC treatment (n = 8, range 14–77 days; Figure 7B) with T-ALL (Figures 7G and 7K). All but one *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/+}$ mouse died by 80 days after pIpC treatment (n = 6, range 26–115 days; Figure 7B) with T-ALL (data not shown). Nine of twelve *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice survived for 115 days (n = 12, range 19–115 days; Figure 7B) with myeloproliferative disorder in the spleen (Figure 7I) but no evidence of T-ALL in the spleen or thymus (Figures 7I and 7M). Spleens from *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice were larger than spleens from control or *Mx1-Cre; Pten*$^{fl/+}$*; Rictor*$^{fl/fl}$ mice (Figures 7C and 7E). In contrast, thymuses from *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice were not enlarged relative to control or *Mx1-Cre; Pten*$^{fl/+}$*; Rictor*$^{fl/fl}$ mice (Figures 7D and 7E). The *Mx1-Cre; Pten*$^{fl/fl}$*; Rictor*$^{fl/fl}$ mice that died in this experiment did have T-ALL. However, genotyping of genomic DNA from one T-ALL revealed

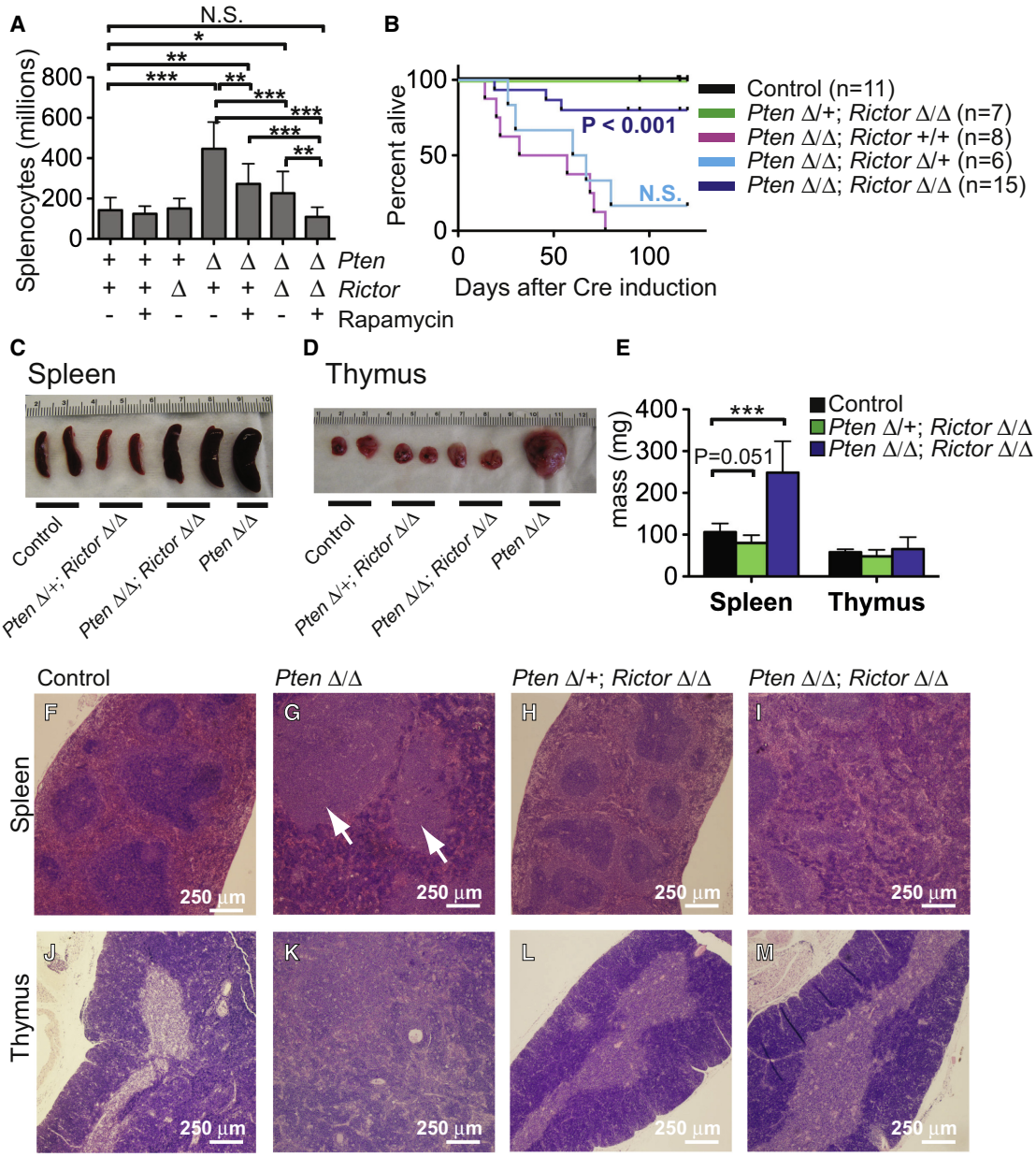

Figure 7. *Rictor* Deficiency Reduced the Severity of Myeloproliferative Disorder and the Incidence of Leukemia after *Pten* Deletion

(A) Two weeks after pIpC treatment in adult mice, spleen cellularity increased significantly in *Mx1-Cre; Pten*$^{fl/fl}$ (*Pten*Δ/Δ) mice due to myeloproliferative disorder, and this increase was significantly attenuated by *Rictor* deletion and rapamycin treatment (n = 5–10 mice/genotype, *p < 0.05; **p < 0.01; ***p < 0.001; N.S., not significant [p > 0.05]).

(B) Kaplan-Meier survival curves after pIpC treatment showed that *Rictor* deletion significantly increased the survival of *Pten*-deleted mice (p < 0.001 relative to *Pten*Δ/Δ; *Rictor*+/+ mice by log-rank test).

(C–E) Spleen and thymus sizes of mice from the same experiment 16 weeks after pIpC treatment (E; n = 5–7 mice/genotype, ***p < 0.001).

(F–M) A myeloproliferative disorder was evident in the spleens of *Pten*Δ/Δ; *Rictor*Δ/Δ mice (I) but not control or *Pten*Δ/+; *Rictor*Δ/Δ mice (F and H). T-ALL was observed in the spleens of *Pten*Δ/Δ; *Rictor*+/+ mice (G, arrows) but not in the spleens of other genotypes. Thymuses of control (J), *Pten*+/+; *Rictor*Δ/Δ (L), and *Pten*Δ/Δ; *Rictor*Δ/Δ (M) mice did not have T-ALL, in contrast to *Pten*Δ/Δ; *Rictor*+/+ thymuses (K).

that the floxed *Rictor* allele had not been deleted in this clone of leukemia cells (data not shown). *Rictor* deletion thus significantly reduced the incidence of leukemia in mice after *Pten* deletion. PTEN is therefore required in adult, but not neonatal, hematopoietic cells to suppress leukemia by reducing mTORC2 activation.

DISCUSSION

The PI3-kinase pathway regulates adult HSC proliferation and leukemogenesis. *Akt1/2* deletion increases adult HSC quiescence (Juntilla et al., 2010). Increased activation of the

PI3-kinase pathway by *Pten* deletion, overexpression of *Akt1*, or *Tsc2* deletion promotes adult HSC proliferation and HSC depletion (Yilmaz et al., 2006; Zhang et al., 2006; Chen et al., 2008; Gan et al., 2008; Kharas et al., 2010; Lee et al., 2010). However, fetal and neonatal HSCs/MPPs did not exhibit increased AKT phosphorylation relative to adult HSCs/MPPs even though more fetal/neonatal HSCs/MPPs were in cycle (Figures 1B–1E). This suggests that while increased PI3-kinase pathway activation by *Pten* deletion can drive adult HSCs into cycle, sustained AKT phosphorylation is not observed in HSCs that divide frequently under physiological conditions. Consistent with this, *Rictor* deletion reduced AKT phosphorylation (Figures 4M and 4N) but had only modest effects on the maintenance and function of HSCs (Figure 5). Nonetheless, *Rictor* was required for increased HSC proliferation (Figure 6C), HSC depletion (Figures 6G–6K), and leukemogenesis (Figure 7) following *Pten* deletion. PTEN is therefore required in adult, but not neonatal, hematopoietic cells to maintain HSCs and to suppress leukemia by reducing mTORC2 activation. *Rictor* deletion also reduces the incidence of prostate cancer after *Pten* deletion, while having little effect on wild-type prostate epithelium (Guertin et al., 2009).

Our data indicate that mTORC1 and mTORC2 have distinct functions in dividing HSCs. While *Rictor*-deficient HSCs gave long-term multilineage reconstitution of irradiated mice (Figure 5), Kalaitzidis et al. (2012) found that *Raptor*-deficient HSCs were unable to reconstitute irradiated mice. Fetal HSCs and cyclophosphamide/G-CSF mobilized HSCs exhibited increased mTORC1, but not mTORC2, activation relative to quiescent adult HSCs (Figures 1D and 1G). Rapamycin treatment, but not *Rictor* deletion, reduced HSC mobilization after cyclophosphamide/G-CSF treatment (Figure 6F). These results demonstrate that mTORC1 is more important than mTORC2 for the proliferation of HSCs under physiological conditions.

In contrast, both mTORC1 and mTORC2 were activated by *Pten* deletion (Figure 1D). *Rictor* deletion, but not rapamycin treatment, reduced HSC mobilization after *Pten* deletion (Figure 6D). Rapamycin treatment and *Rictor* deletion both reduced HSC proliferation after *Pten* deletion (Figure 6C) and additively reduced the severity of myeloproliferative disorder following *Pten* deletion (Figure 7A). These results demonstrate that mTORC2 is a major mediator of the effects of *Pten* deletion on hematopoietic cells, and that mTORC2 likely signals through mTORC1-dependent and mTORC1-independent pathways. Therefore both mTORC1 and mTORC2 contribute to the hematopoietic phenotypes observed after *Pten* deletion, consistent with our previously published results (Yilmaz et al., 2006; Lee et al., 2010) and the results in Kalaitzidis et al. (2012).

Our data indicate that temporal changes in the regulation of the PI3-kinase pathway lead to temporal changes in the mechanisms that regulate HSC function and leukemogenesis. This likely explains why conditional deletion of *Pten* from fetal hematopoietic cells using *VE-Cadherin-Cre* does not lead to the development of leukemia until around 8 weeks after birth (Guo et al., 2008). It is not yet clear why *Pten* deletion increases PI3-kinase pathway activation in adult, but not neonatal, HSCs. Neonatal HSCs were capable of activating the PI3-kinase pathway when cultured (Figure 4D), but exhibited a cell-autonomous difference relative to adult HSCs in their dependence upon PTEN in transplantation assays (Figures 2E–2H). We have identified a number of gene products that are differentially expressed between neonatal and adult HSCs and that could potentially influence PI3-kinase pathway signaling (Figure S2). However, much more work will be required to assess their functions in neonatal and adult HSCs.

Since neonatal HSCs and other hematopoietic cells can harbor mutations in *Pten* and *p53* without transforming into leukemia until adulthood, our data suggest that mutated cells may persist for years in children before a change in developmental context renders these mutations competent to induce leukemia. Prolonged persistence of cells with leukemogenic mutations has been documented in human pediatric leukemia patients (Wiemels et al., 1999a, 1999b). In the past, this latency has been attributed to the time necessary to accrue secondary mutations. However, our data suggest an additional explanation: that some of these mutations were not competent to cause leukemia until the blood cells matured into a susceptible developmental context. This could explain why T-ALL presents more commonly in older rather than younger children. Our findings raise the possibility of reprogramming therapies that are analogous to differentiation therapies. Reprogramming of adult hematopoietic cells to a fetal or neonatal identity (Kim et al., 2007; He et al., 2011) could blunt the effects of leukemogenic mutations that require an adult developmental context to induce neoplastic proliferation.

EXPERIMENTAL PROCEDURES

Mouse Strains and pIpC Injections

Pten$^{fl/fl}$ mice, *Mx1-Cre* mice, and *p53*$^{-/-}$ mice have been previously described (Jacks et al., 1994; Kühn et al., 1995; Groszer et al., 2006). These mice were all backcrossed for at least eight generations onto a C57BL/6Ka-Thy-1.1 (CD45.2) background. See the Supplemental Experimental Procedures for a description of the generation of *Rictor*^{fl} allele. Expression of *Mx1-Cre* was induced by a single subcutaneous injection of pIpC (Amersham) in neonatal mice at P2 or by three intraperitoneal injections over 6 days in adult mice beginning 6 weeks after birth. pIpC dose was determined empirically for each lot so that >95% of HSC colonies exhibited complete recombination of the *Pten*$^{fl/fl}$ and *Rictor*$^{fl/fl}$ alleles (Figures S1 and S4). Neonatal mice received 1–2 μg/dose, depending on pIpC lot. Adult mice received 10 μg/dose. All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan (UM), where these studies were initiated, or at the Animal Resource Center at UT-Southwestern Medical Center, where the studies were completed. All animal procedures were approved by the UM and UT Southwestern Committees on the Use and Care of Animals.

Flow Cytometry and Isolation of HSCs

Bone marrow cells and splenocytes were obtained, stained, and analyzed by flow cytometry as previously described (Lee et al., 2010; Nakada et al., 2010). Antibodies and detailed methods are in the Supplemental Experimental Procedures (Table S1).

BrdU Incorporation and Cell Cycle Analysis

BrdU (Sigma; diluted to 5–10 mg/ml in PBS) was administered by IP injections (100 mg/kg/dose) given every 8 hr beginning 24 hr prior to bone marrow harvest. HSCs were stained and enriched by c-kit selection as described in the Supplemental Experimental Procedures, and BrdU incorporation was measured by flow cytometry using the APC BrdU Flow Kit (BD Biosciences). For cell cycle analysis, HSCs were stained with antibodies against CD150, CD48, lineage markers, c-kit, and Sca-1. c-kit^{+} cells were enriched by selection with paramagnetic beads (Miltenyi Biotec, Auburn, CA). The enriched cells were fixed and permeablized with cytofix/cytoperm buffer (BD Biosciences) then washed in staining medium, and stained with DAPI (20 μg/ml). HSCs were analyzed by flow cytometry. For analysis of AKT phosphorylation in

HSCs/MPPs in different phases of the cell cycle (Figure 1E), Vybrant DyeCycle Violet Stain (Invitrogen) was used to analyze DNA content while preserving cell viability. A detailed protocol is provided in the Supplemental Experimental Procedures.

Western Blots

Twenty or thirty thousand CD48$^-$LSK cells were sorted and then resorted (to ensure purity) into Trichoracetic acid (TCA), and the volume was adjusted to a final concentration of 10% TCA. LSK cells were sometimes incubated at 37°C in Iscove's medium plus 2% fetal bovine serum for 30 min prior to protein extraction in 10% TCA. Extracts were incubated on ice for at least 15 min and centrifuged at 16,100 × *g* at 4°C for 10 min. Precipitates were washed in acetone twice and dried. The pellets were solubilized in 9M urea, 2% Triton X-100, and 1% DTT. LDS loading buffer (Invitrogen) was added and the pellet was heated at 70°C for 10 min. Samples were separated on Bis-Tris polyacrylamide gels (Invitrogen) and transferred to PVDF membrane (Millipore). Antibodies are listed in the Supplemental Experimental Procedures (Table S1). Western blotting was performed according to the protocol from Cell Signaling Technologies, and blots were developed with the SuperSignal West Femto chemiluminescence kit (Thermo Scientific). Blots were stripped (1% SDS, 25 mM glycine [pH 2]) prior to reprobing.

Long-Term Reconstitution Assays

Competitive reconstitution experiments were performed as previously described (Lee et al., 2010; Nakada et al., 2010). A complete description of long-term reconstitution assays is provided in the Supplemental Experimental Procedures.

Survival and Leukemia Analysis

After administering pIpC to mice as described above, the mice were monitored daily and morbid mice were euthanized. Spleen and thymus tissues were fixed in buffered 10% formalin and embedded in paraffin for sectioning and hematoxylin and eosin (H&E) staining. Bone marrow cytospin specimens were Wright-Giemsa stained (Sigma). J.A.M., a pediatric hematologist, reviewed all slides and bone marrow specimens.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes five figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.05.026.

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